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CONTRACT NO: DAMD17-90-C-0094

TITLE: TRANSDOMINANT REV AND PROTEASED MUTANT PROTEINS OF
         HIV/SIV AS POTENTIAL ANTIVIRAL AGENTS IN VITRO AND
         IN VIVO (AIDS)

PRINCIPAL INVESTIGATOR: Flossie Wong-Staal, Ph.D.

CONTRACTING ORGANIZATION: University of California, San Diego
                            Department of Medicine, 0665
                            La Jolla, California   92093-0665

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19. ABSTRACT (Continue on reverse if necessary and identify by block number)
The major goal of this contract is to use gene therapy to target essential genes of HIV/SIV in order to inhibit virus expression. Our initial focus was to generate transdominant mutants of rev and protease genes and to evaluate them in an in vivo model. During the last year, we have expanded our approaches to include use of antisense oligodeoxynucleotides (ODN) directed to the Rev Response Element (RRE) and ribozymes that target viral mRNAs. These latter approaches have yielded very encouraging positive data. Binding of antisense ODN to RRE was optimized according to sequence and in vitro reaction. We demonstrated that this binding indeed interferes with the normal interaction of the Rev protein as well as an important cellular factor (NF_{RRE}) with RRE RNA, and furthermore, phosphorothioate derivative of the antisense ODN efficiently inhibited virus replication. In parallel, a hairpin ribozyme targeting the leader sequence of the HIV genome was shown to inhibit virus expression in a target specific manner. Applications of these approaches will be pursued in the in vivo SCID mouse model in the coming year.

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FOREWORD

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_____In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

_____For the protection of human subjects, the investigator(s) have adhered to policies of applicable Federal Law 45 CFR 46.

__✓__In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

PI Signature _____ Date _10/14/92_

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QUARTERLY REPORT

1. Contract No. __DAMD17-90-C-0094__ 2. Report Date __Oct. 14, 1992__

3. Reporting period from __March 4, 1992__ to __June 3, 1992__

4. PI __Flossie Wong-Staal, Ph.D.__ 5. Telephone No. __(619) 534-7958__

6. Institution __University of California, San Diego__

7. Project Title: __Transdominant Rev and Protease Mutant Proteins of__
__HIV/SIV as Potential Antiviral Agents in Vitro and in Vivo.__

8. Current staff, with percent effort of each on project.

Flossie Wong-Staal, PhD	6.00%	Randy Talbott	100.00%
Joshua Ojwang, PhD	53.00%		
Modem Suhasini, PhD	45.00%		

9. Contract expenditures to date:

Personnel	23,903.12	Supplies	145.94
Travel	-0-	Other	577.64
Equipment	-0-		
		TOTAL	24,626.70

10. Comments on administrative and logistical matters.

11. Use additional page(s), as necessary, to describe scientific progress for the quarter in terms of the tasks or objectives listed in the statement of work for this contract. Explain deviations where this isn't possible. Include data where possible.

12. Use additional page(s) to present a brief statement of plans or milestones for the next quarter.

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Modem Suhasini, PhD	55.00%		

9. Contract expenditures to date:

Personnel	22,545.75	Supplies	-0-
Travel	1,740.35	Other	104.42
Equipment	-0-		
		TOTAL	24,390.52

10. Comments on administrative and logistical matters.

11. Use additional page(s), as necessary, to describe scientific progress for the quarter in terms of the tasks or objectives listed in the statement of work for this contract. Explain deviations where this isn't possible. Include data where possible.

12. Use additional page(s) to present a brief statement of plans or milestones for the next quarter.

I. Introduction

As we enter the second decade of the AIDS pandemic, we see a sharp increase in HIV seroprevalance in the developing countries of Africa, Latin American, and Asia, with no clear indication of decline in the developed countries. As a result, large increases in AIDS cases and related mortality are expected in the next decade, particularly in the developing countries. As yet, there is no effective treatment or vaccine for AIDS, although the reverse transcriptase inhibitors such as AZT and ddI have been shown to prolong life in some patients and a number of candidate drugs and vaccines are poised to go into clinical trials. Continued efforts to identify new strategies and targets and to refine those being developed are critically needed, since a "magic bullet" is not likely to be found in the battery of candidate drugs and vaccines already available. Furthermore, a combination treatment regiment using multiple drugs may be indicated to counter the problems of toxicity and resistance.

In theory, inhibition of HIV-1 replication should be achievable by interfering with various key steps in the virus life cycle, including virus entry, reverse transcription, transcription, transactivation, translation, packaging, or release of virus particles. Although we initially focused on generating transdominant mutants of rev and protease genes, slow or unsatisfactory outcomes of these efforts stimulated to explore additional avenues for gene therapy against HIV. The diverse approaches that have been taken include antisense and ribozyme technologies. Both approaches target the same step in the HIV-1 viral life cycle, the utilization of viral mRNA (reviewed in Ref. 1). Conventional antisense RNAs and DNAs have been shown to impair gene expression and may have utility as antiviral and anticancer agents.

One important theme to emerge from the study of HIV regulation is the importance of RNA-Protein interactions, not only between Tat and Rev and their respective targets, TAR and RRE, but also TAR and RRE binding proteins as well as Tat and Rev binding proteins. These components form large functional complexes, the disruption of which would halt virus production. The use of RNA decoys, transdominant mutant proteins and anti-sense/catalytic RNAs all aim at interfering such complex formation. We have utilized antisense to RRE as a means to inhibit Rev function, and a hairpin ribozyme targeting the leader sequence to cleave HIV mRNAs, including tat and rev mRNA.

Our objectives are as follows:

1. To drive optimal antisense oligonucleotides against HIV-1 RRE that will inhibit Rev-RRE and NF_{RRE}-RRE interactions, as well as HIV replication.

2. To design and construct ribozymes targeting conserved sequences of HIV-1 RNA.

3. To evaluate the capacity of the ribozymes to inhibit replication of HIV in transient transfection systems.

4. To insert ribozymes in amphotropic murine retrovirus vector and optimize expression in human T-cells using different internal promoters.

5. To transduce PBL cells or neoplastic T-cell lines with retrovirus expressing the ribozymes to determine if uninfected cells could be protected from de novo infection and if chronically infected cells would be repressed in virus production.

6. To determine if SCID-Hu mice treated with antisense oligonucleotides or reconstituted with human PBL transduced by ribozyme-expressing retrovirus vector would be refractory to HIV infection and/or virus induced CD4 cell depletion.

We have made good progress on specific aims 1-3 and will extend into specific aims 4-6 in the coming year.

II. Progress

A. Inhibition of HIV-1 by antisense Oligonucleotides directed against Rev Responsive Element (RRE).

Structural and genomic RNA transcripts of the human immunodeficiency virus contain a highly structured motif known as the Rev-responsive element (RRE). The viral Rev protein and a cellular nuclear factor, NF_{rre}, bind specifically to RRE RNA, positively regulating expression of Env, Gag, and Pol proteins (2). This unique regulatory scheme represents an attractive target for inhibition of viral expression. We examined various antisense oligonucleotides (ODN) directed against the RRE for their ability to bind stably to RRE, to abolish or reduce the binding of Rev and NF_{rre} proteins, and to inhibit the expression of HIV-1 p24 in HIV transfected cells.

Sequence optimization of Antisense Oligonucleotides that binds RRE. Phosphodiester and phosphorothioates targeted to the HIV-1 RRE were designed (Figure 1A) to target the stem 1 and stem loop 2 region of RRE, which encompasses the Rev and NF_{RRE} binding sites. These oligomers were designed to go over the bulge of the stem 1 (i.e., aRRE1), to cover half the stem loop 2 (aRRE2, aRRE, aRRE4). This approach has been shown to disrupt the secondary structure of some mRNA messages (3). Internally labeled RRE was transcribed in vitro from T7 promoter driven vector. Unlabeled oligonucleotides were added either at the time of addition of ribonucleotide or well after transcription was completed. Oligonucleotides which disrupt the secondary structure of the stably-folded RRE RNA were expected to bind whether added before or after transcription, while those incapable of disrupting the structure of folded messages were expected to bind only when added at the time of transcription. Gel shift experiments showed that oligonucleotides aRRE1, aRRE2, and aRRE3 regions could bind with specificity regardless of whether the RNA was first allowed to fold (Figure 1B). However, only antisense ODN which included regions which hybridized with at least half of the unpaired bases in loops or bulges of the RRE showed binding to prefolded RNA structure (data not shown). The binding of aRRE1 was most striking, and subsequent experiments were focused on this oligonucleotide.

To optimize the sequence for RRE binding, we designed a series of 24-mer ODN's 1A-F (Figure 2A), staggering three-base steps towards the 5'-end of RRE RNA. As seen in Figure 2B, moving the ODN target as little as 6 bases toward the 5'-

end of RRE RNA. As seen in Figure 2B, moving the ODN target
as little as 6 bases toward the 5' end of the RRE reduced the
degree of binding by 2-3 fold. Titration experiments showed
that 50% of RRE RNA was found (shifted) at an oligonucleotide
concentration between 0.3 pmole and 1.0 pmole (data not
shown), corresponding to approximately 1 copy of oligomer for
every 1.5-2 copies of RRE RNA.

Antisense ODN rendered RRE RNase H sensitive.
The efficiency of oligonucleotide in inhibition of mRNA
translation may largely dependent on the cleavage of
mRNA:oligonucleotide hybrid by endogenous ribonuclease-H
(RNase-H) (4). RNase-H recognizes DNA:RNA hybrid and cleaves
only the RNA strand. We examined if aRRE1 is able to induce
RNase-H cleavage of RRE. Both the phosphorothioate and
normal aRRE1 lead to the degradation of the transcript as
shown in Figure 3, lanes 3 and 4. Therefore it does induce
RNAse H Activity. However, phosphorothioate aRRE2 did not
induce the RNAse-H activity. The observation may partly be
due to the inability of modified aRRE2 to bind the RRE RNA
under these experimental conditions. It has been previously
shown that modification of ODN reduce their binding ability
(5).

Effect OF aRRE1 on Rev and NF$_{RRE}$ Binding.
Given that the specific interaction of Rev and RRE is
critical in the HIV replication cycle, it was important to
study the effect of aRRE1 on Rev-RRE interaction. The
addition of a RRE1 induced a shift of the RRE probe (Figure
4A, lane 5) and completely inhibited Rev binding (lane 6).
The addition of other antisense oligomers or mutated control
oligomers had no effect, implying the binding and inhibition
was specific to aRRE1. Prediction of the structure of RRE
bound to aRRE1 suggests that this may be due to extensive
perturbation of RRE stem1 structure. Degradation of the
probe in lane 6 could be due to contaminating RNAse-H
activity in the Rev protein preparation.

NF$_{RRE}$ is a 50-55 Kd cellular protein which binds specifically
to RRE RNA and Rev as a complex (6). Recent data in our
laboratory suggest that NF$_{RRE}$ plays a positive role in Rev
transactivation (Y.N. Vaishnav, et al., unpublished). As
shown in Figure 4B, binding of the NF$_{RRE}$ was substantially
reduced in the presence of aRRE1 (lane 6).

***In vitro* Antiviral Activity of aRRE2 S-ODN.**
In order to determine sequence specific antiviral activity of
antisense ODN, we utilized a system where virus expression
independent of early infection events was monitored. Hela-
LTR-LacZ "Magic" cells were transfected with the HXB2 genomic
DNA followed by ODN. The inhibition of expression of HIV-1
p24 was used to determine the effectiveness of the ODNs as as
inhibitors of HIV-1 replication and expression. As seen in

Figure 5, both S-aRRE1 and S-aRRE2 were capable of significant inhibition (84-95%) of HIV-1 expression in a dose dependent fashion (even at concentrations as low as 2.5 μM).

B. **Inhibition of HIV Replication by a Hairpin Ribozyme**

Ribozymes are RNAs that possess the dual properties of RNA sequence-specific recognition analogous to conventional antisense molecules, and RNA substrate destruction via site-specific cleavage. The cleavage reaction is catalytic in that more than one substrate molecule is processed per ribozyme molecule. We have designed a hairpin ribozyme that cleaves HIV-1 RNA in the leader sequence (at nucleotide +111/112 relative to the transcription initiation site). The ribozyme was tested for *in vitro* cleavage of target RNA, and for antiviral efficacy in vivo. The results suggest that this HIV-1 directed hairpin ribozyme may be useful as a therapeutic agent.

Specific cleavage of HIV-1 RNA by hairpin ribozyme *in vitro*. A hairpin ribozyme was engineered to cleave a site in the 5' LTR of the HXB2 clone of HIV-1 (Figure 6A). The target sequence UGCC C*GUC UGUUGUGU (with cleavage occurring at the*) is highly conserved among all HIV-1 isolates. The hairpin ribozyme was engineered such that it could base pair to the two sequences flanking the C*GUC to form helices 1 and 2 (Figure 6B). Heliz 2 is fixed in length at four bases by the functional requirements of the hairpin ribozyme, however the length of helix 1 could be varied. The length of helix 1 which provided optimal catalytic activity was experimentally determined to be 8 nt (Hampel, unpublished observation).

In vitro this ribozyme cleaved the target substrate with efficiency as determine by its kinetic parameters (not shown). Under very mild reaction conditions (37°, pH 7.5, 12mM MgC12, 40mM Tris and 2 mM spermidine), the kinetic constants were as follows: Km=100 nM and Kcat-1.6/min. This gives an enzyme efficiency of Kcat/Km = 0.016 nM-1min-1 as compared to 0.07 for the original native hairpin ribozyme (7). The catalytic efficiency, relative to the original tobacco ringspot ribozyme, is therefore 23%. A disabled ribozyme was prepared with the same sequence as Figure 1B except nucleotides 22-AAA-24 (Figure 6C) were changed to 22-CGU-24 (Figure 6D). Although, this RNA still binds to the target sequence, no catalytic activity could be detected *in vitro* (data not shown).

Effects of Ribozyme on HIV-1 Expression in a Transient Assay. The practical application of ribozymes as therapeutic agents *in vivo* will depend on their ability to function in a complex cellular environment. This requires stable expression of the ribozyme in the cell, specificity

for the target RNA, accessibility of mRNA targets for cleavage and lack of cytotoxicity of the endogenously expressed ribozyme. To address these issues, the sequences containing the HIV directed ribozyme and its disabled counterpart were cloned into mammalian expression vectors under the control of the human ß-actin promoter and the PolIII promoters for tRNA$_{val}$ and adenovirus VA1 (Figure 7). The resultant plasmid DNAs were used in transfection experiments in HeLa cells. The pHXB2gpt plasmid and an LTR-CAT plasmid were co-transfected. In this assay, the pHXB2gpt plasmid provides the target mRNA which is translated into viral proteins, including Tat. Tat protein in turn transactivates the CAT-linked LTR promoter (pC15-CAT) (Figure 8). Ribozyme activity is measured by the level of reduction of tat transactivation and gag expression. As shown in Figure 8, the expression of HIV-1 ribozyme in this transient assay inhibited HIV-1 expression and virus production significantly (~70-90%). As a control, tat-expressing plasmid which lacks the target sequence was not inhibited by the ribozymes. In addition, the disabled ribosomes did not inhibit CAT activity or gag expression (not shown), suggesting that the wildtype ribozymes functioned catalytically, and not as antisense molecules.

III. Conclusions

As indicated in the first annual report, we had planned to diversify our gene therapy approach against HIV. In this report, we present encouraging data on utilizing two novel approaches in inhibiting HIV replication: (a) we showed that antisense deoxynucleotides targeting the RRE sequence can disrupt binding of RRE to Rev and the cellular factor, NF_{RRE}, and inhibit HIV expression in a sequence specific manner. (b) a hairpin ribozyme targeting the leader sequence of HIV-1 inhibits HIV expression in a manner which is dependent on presence of target sequence and a catalytically active RNA. Our current aims are to (i) optimize the delivery and dosage of the antisense ODN for PBL's and additional HIV isolates. (ii) optimally expression the ribozyme in a retrovirus vector; and (iii) to evaluate these approaches in the SCID-Hu PBL mouse model.

IV. References

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V. FIGURE LEGENDS

Figure 1. <u>Design of antisense ODN directed against the HIV-1 Rev responsive element (RRE)</u>.

(A) The minimum energy predicted secondary structure of RRE and designed antisense oligomers directed against different portions of the RRE RNA (sequences given in the box).
(B) The binding of anti-RRE oligomers to internally labeled RRE RNA: RRE RNA only (*lane* 1) RRE RNA + aRRE1 (*lane* 2), RRE RNA + aRRE2 (*lane* 3), RRE RNA - aRRE3 (*lane* 4), RRE RNA + aRRE4 (*lane* 5), RRE RNA - aRRE5 (*lane* 6), and RRE RNA + maRRE1=mRRE1[mutant form of aRRE1] (*lane* 7).

Figure 2. <u>Sequence Optimization of aRRE1</u>.

(A) Three-base step walk towards the 5'-end of RRE RNA starting with aRRE1 ODN, complementary to bases 7378-7402 (24 nucleotides) and ending with aRRE1F ODN complementary to bases 7359-7383. The mRRE1 is a mismatched ODN corresponding to aRRE1 used as control. These are all phosphodiester oligonucleotides. **(B)** Binding of stem loop I ODNs to RRE RNA: RRE RNA only (*lane* 1), RRE RNA = aRRE1 (*lanes* 2 and 3), RRE RNA + aRRE1A (*lane* 4), RRE RNA + aRRE1B (*lane* 5), RRE RNA + aRRE1C (*lane* 6), RRE RNA +aRRE1D (*lane* 7), RRE RNA + aRRE1E (*lane* 8) and RRE RNA + aRRE1F (*lane* 9). M represents multimer forms of RRE RNA.

Figure 3. <u>Sensitivity of RRE-ODN to RNase-H</u>.

One unit of RNase-H was added to prehybridized mixture of RRE RNA (0.3 pmoles) and normal and/or phosphorothioate deoxyoligomers (10 pmoles) in final volume of 10 ml and incubated at 37°C for 2 hours.

Figure 4. <u>Effects of aRRE1 ODN Rev-RRE and NF$_{RRE}$-RRE Interactions</u>.

Binding of labeled RRE to (A) the Rev protein and (B) NF$_{RRE}$ was carried out according previously described conditions (6).

Figure 5. <u>Antiviral activity of anti-RRE phosphorothioates oligonucleotides</u>.

Hela CD4 cells were first transfected with the pHXB2 DNA, and after three hours, with the phosphorothioated ODN.

Figure 6. <u>Structure of hairpin ribozyme</u>.

(A) The sequence and location of the target site in the HIV-1 genome. Cleavage occurs at the arrow between nucleotides + 111/112 relative to the transcription initiation site (HIV-1$_{HXB2}$). The 16 base target site is found in the leader of all known HXB2 mRNA species.

(B) The hairpin ribozyme used to cleave the HIV-1 substrate RNA. Substrate RNA (SHIV) has the target sequence shown plus additional gcg sequence at it 5'end. Hairpin catalytic RJA (RHIV) having the "hairpin" catalytic motif is designed as shown. It also has additional vector sequence shown in lower case letters.

(C) Wild-type (from plasmid pß-dHR) and disabled ribozyme.

(D) The 5' cap site and 3' terminus with 2', 3' cycic phosphate are depicted.

Figure 7. <u>Expression of ribozyme from eukaryotic promoters</u>.

The ribozyme was expressed from both polII (ß-actin) and polIII (tRNA$_{Val}$ and VA1) promoters.

Figure 8. <u>Effect of ribozyme on HIV-1 expression in a transient assay</u>.

The expression of this plasmid in cells provides the ribozyme target. The effector plasmid (pHXB2gpt) and a reporter plasmid (pC15CAT) were co-transfected into HeLa cells with HIV-1 5' leader sequence-specific reibozyme containing plasmids (pß-HR, pJT-HR, pJV-HR) by calcium phosphate method in a molar ratios of 1:5 or 1:10 (effector plasmid:ribozyme plasmid). The control consisted of HeLa cells transfected with pHXB2gpt, pC15CAT and pHßApr-1 vector lacking the ribozyme sequences. pTat is a tat-expressing plasmid driven by the SV40 late promoter and lacks the ribozyme target sequence. After 48 hours the cells were harvested and the supernatant was subjected to p24 antigen analysis, or the cell lysate subjected to CAT enzyme assay. The experiments were repeated three or more times and both values were represented as relative percentages.

Figure 1A
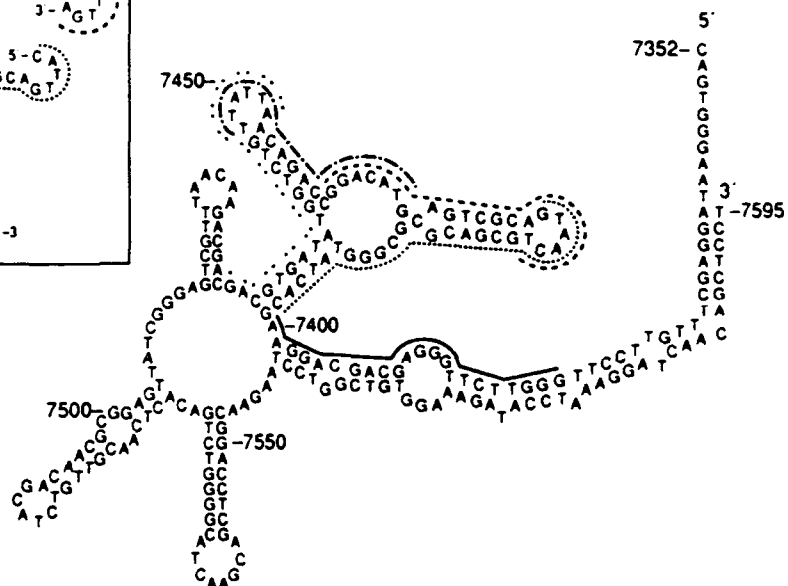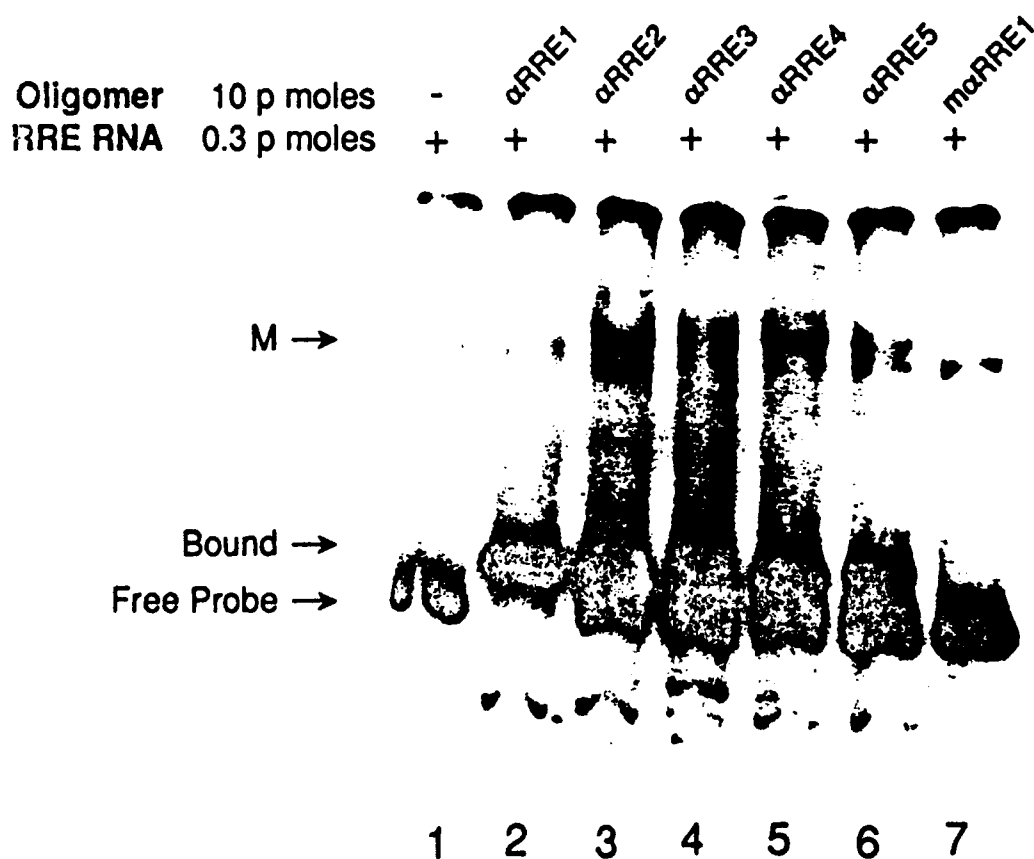
The HIV-1 <u>rev</u> responsive element (RRE)

αRRE4

αRRE5

αRRE3

αRRE2

αRRE1

7352–

3'–7595

7450–

7400

7500–

7550

Figure 1B

Oligomer	10 p moles	-	αRRE1	αRRE2	αRRE3	αRRE4	αRRE5	mαRRE1
RRE RNA	0.3 p moles	+	+	+	+	+	+	+

M →

Bound →

Free Probe →

1   2   3   4   5   6   7
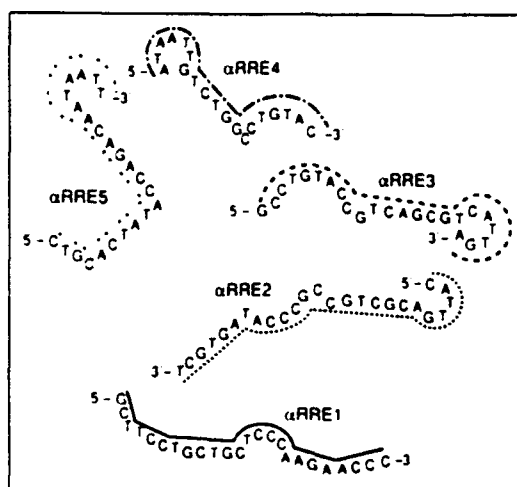
Figure 2A

```
          7402                                    7378
αRRE 1  5'-GCT TCC TGC TGC TCC CAA GAA CCC -3'
   1A        5'-TCC TGC TGC TCC CAA GAA CCC AAG-3'
   1B            5'-TGC TGC TCC CAA GAA CCC AAG GAA-3'
   1C                5'-TGC TCC CAA GAA CCC AAG GAA CAA-3'
   1D                    5'-TCC CAA GAA CCC AAG GAA CAA AGC-3'
   1E                        5'-CAA GAA CCC AAG GAA CAA AGC TCC-3'
   1F                            5'-GAA CCC AAG GAA CAA AGC TCC TAT-3'
```

MαRRE 1 5'-ATT CTC TAT GAT TTC TGG AGG TTT G-3'

Figure 2B
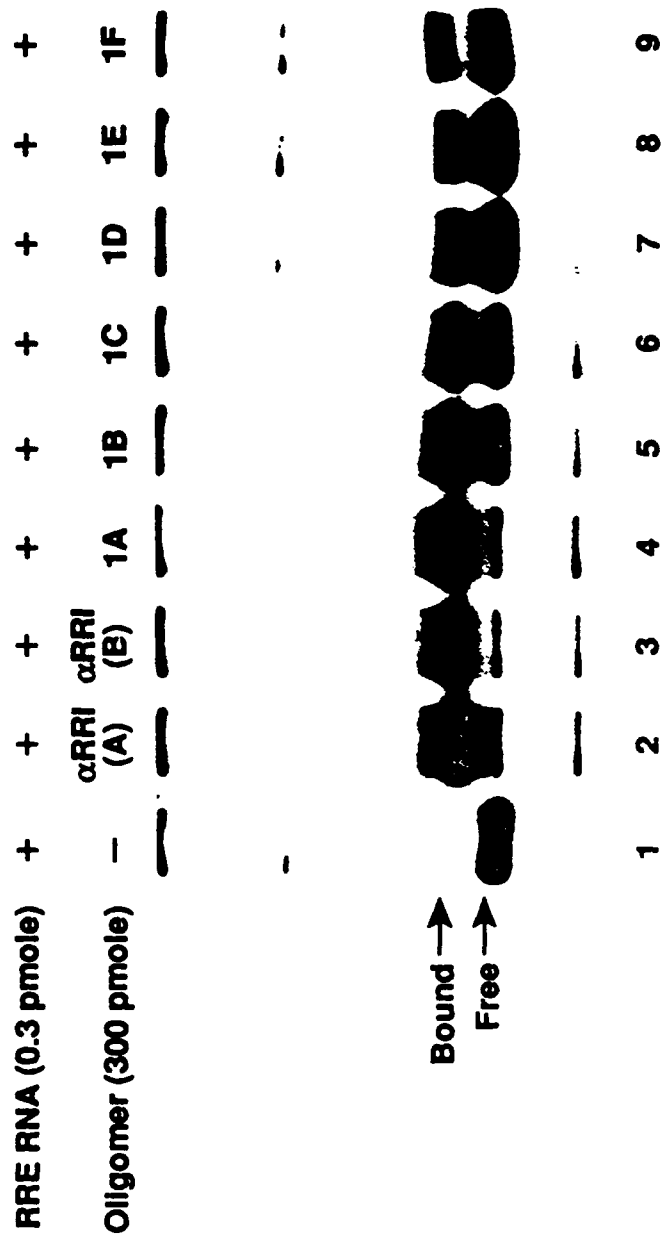
Figure 3

RNA (0.3pmole)	+	+	+	+	+	+
Oligomer (10pmole)		αRRE1	αRRE1	s-αRRE1	s-αRRE2	s-mRRE1
RNase H (3units)	–	–	+	+	+	+

Bound →
Free Probe →
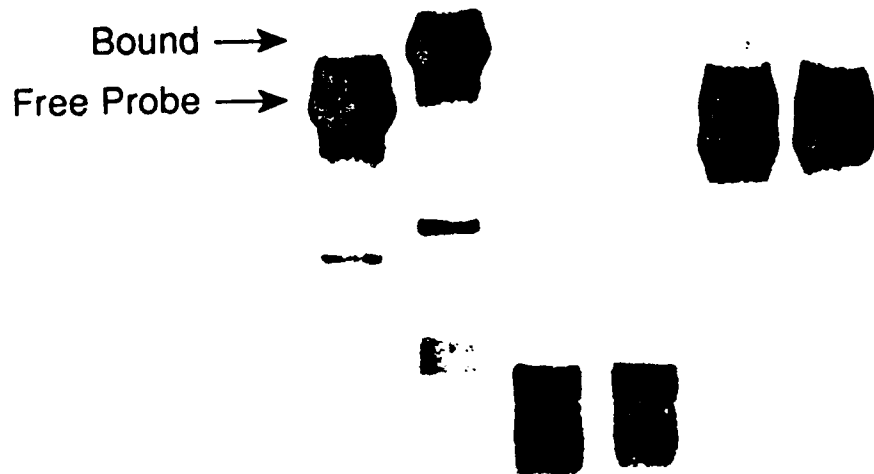
Effect of antisense RRE subdomain
oligos on RRE binding to NF_{RRE}

oligo(1µg)
HNE(6µg)

mαRRE1 αRRE1

− + − + − +

1 2 3 4 5 6

Effect of antisense RRE subdomain
oligos on RRE binding to Rev

oligo(1µg)
Rev(1µg)

mαRRE1 αRRE1

− + − + − +

1 2 3 4 5 6

Figure 5

Inhibition of p24 Antigen Expression
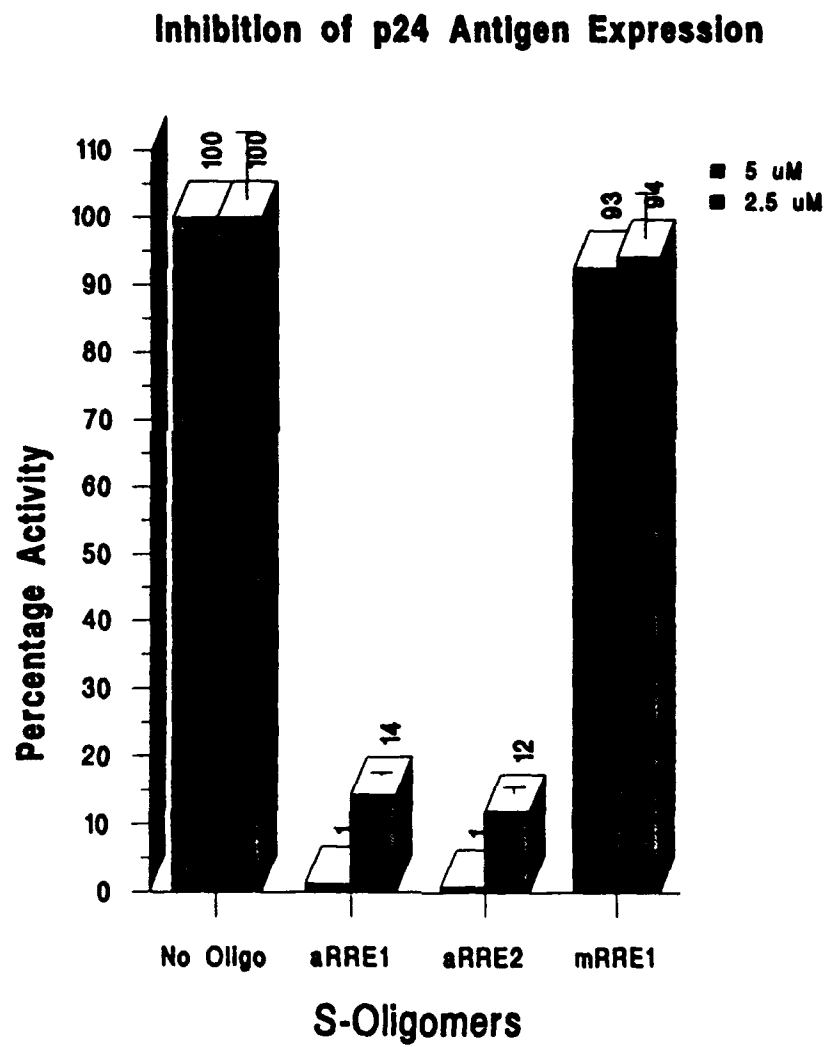
Figure 6A

+111/112

HIV
Substrate RNA
UGCC C GUC UGUUGUGU

5' leader ▪

GAG

POL

LTR

TAT
VIF VPU
VPR
REV

REV
ENV

TAT
NEF

LTR

Figure 6B

HIV Catalytic RNA / Substrate RNA Complex

Catalytic RNA

Site of cleavage

Substrate RNA

3' base
G
C
G

5' base

50 A
U

U UAUAUUA
GUG G 40 C
• • • CUGG
U • • • • • • • •

CAC A GACC A ACGG
30 CAAAG 20
G

GU C
UGCC C C UGUUGUGU 3' base

• • • • • • • • • • • •
A A ACAACACAcugaggg 5' base
10 AG A 1

Helix 4   Helix 3   Helix 2   Helix 1
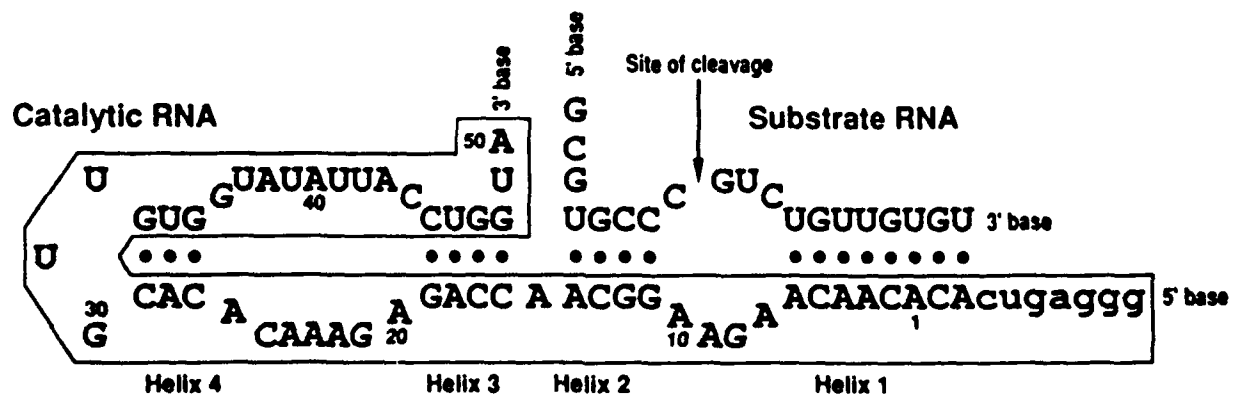
Figure 6C

Figure 6D

FIGURE 7

Cap ribozyme SV40 polyA

Human β-actin promoter

Sal I Hind III

T7 promoter ribozyme

TTTTACTTTTT

Bam H1 Mlu1

tRNA^{Val} promoter

Termination Seq.

T7 promoter ribozyme

TTTTACTTTTT

VAI promoter

Termination Seq.
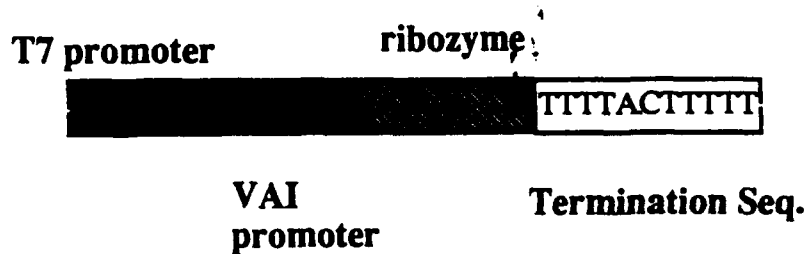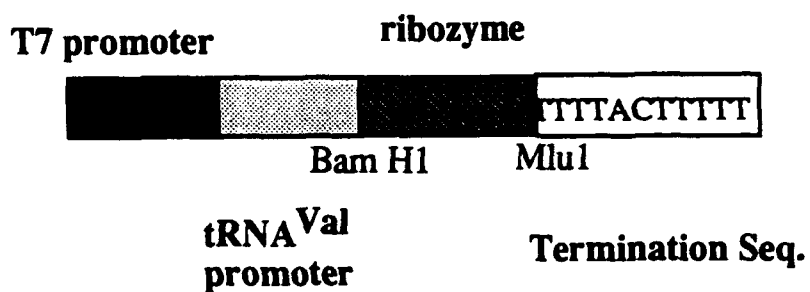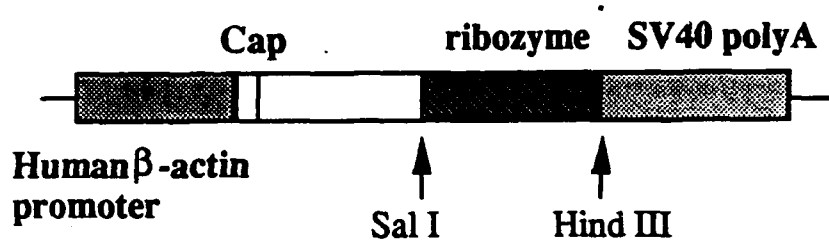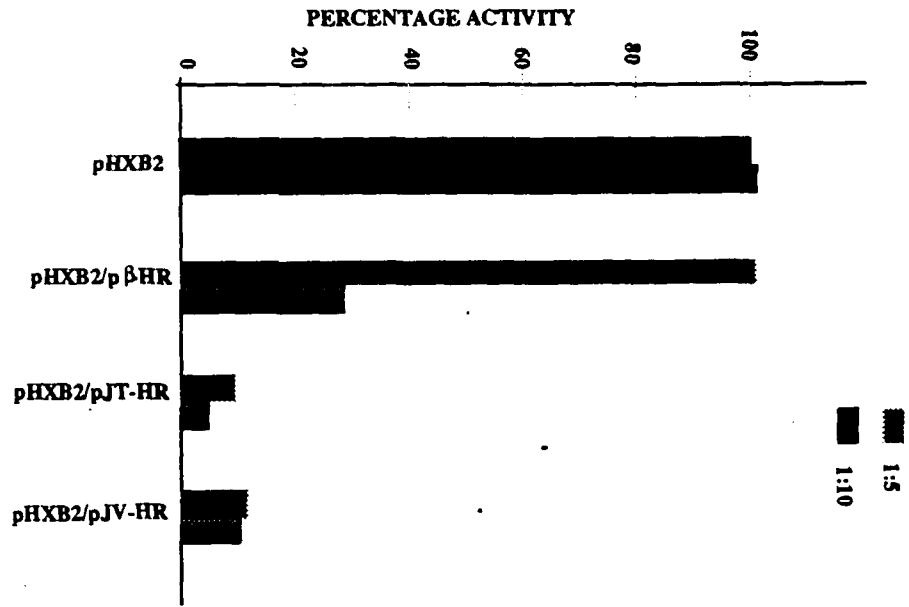
RIBOZYME EFFECT ON p24 EXPRESSION

RIBOZYME EFFECT ON TAT ACTIVITY

FIGURE 8